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Functional Peptide Sequences Derived from Extracellular Matrix Glycoproteins and Their Receptors

Strategies to Improve Neuronal Regeneration

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Abstract

Peptides derived from extracellular matrix proteins have the potential to function as potent therapeutic reagents to increase neuronal regeneration following central nervous system (CNS) injury, yet their efficacy as pharmaceutical reagents is dependent upon the expression of cognate receptors in the target tissue. This type of codependency is clearly observed in successful models of axonal regeneration in the peripheral nervous system, but not in the normally nonregenerating adult CNS. Successful regeneration is most closely correlated with the induction of integrins on the surface of peripheral neurons. This suggests that in order to achieve optimal neurite regrowth in the injured adult CNS, therapeutic strategies must include approaches that increase the number of integrins and other key receptors in damaged central neurons, as well as provide the appropriate growth-promoting peptides in a "regeneration cocktail." In this review, we describe the ability of peptides derived from tenascin-C, fibronectin, and laminin-1 to influence neuronal growth. In addition, we also discuss the implications of peptide/receptor interactions for strategies to improve neuronal regeneration.

Index Entries: Tenascin-C; fibronectin; laminin-1; FN-III repeat; alternatively spliced region; synthetic peptide; receptor; integrin; neurite outgrowth; neurite guidance.

Introduction

Extracellular matrix molecules in the CNS are composed of functional domains that have major actions to regulate neuronal growth. The regions within the protein individually and synergistically provide a number of regulatory cues for neuronal-growth related activities such as migration and adhesion, neurite outgrowth and retraction, and neurite guidance. The diversity of responses to the extracellular matrix may reflect the concerted action of sev-

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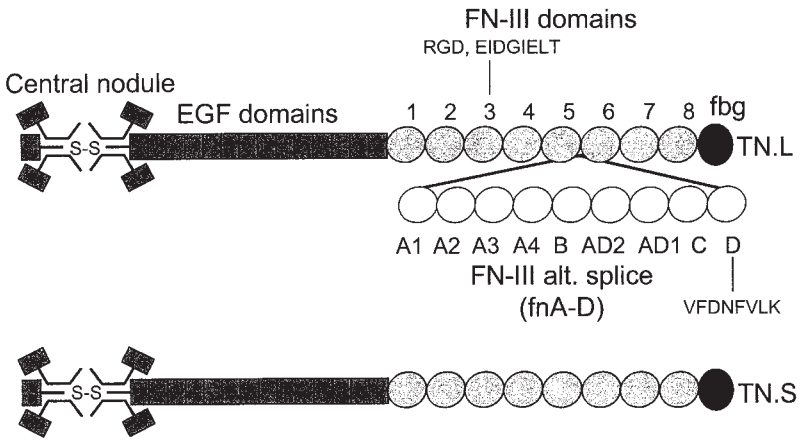

Fig. 1. Multi-domain structure of human tenascin-C. The N termini of three arms are joined to form a trimer, and two trimers are connected via a disulfide bond to form a hexamer. Each arm consists of 14 epidermal growth factor (EGF)-like domains, 8 universal FN-III repeats (fn1-5 and fn6-8), 0-9 alternatively spliced FN-III repeats, and a single fibrinogen (fbg) domain. The largest tenascin-C splice variant (TN.L) contains a series of alternatively spliced FN-III repeats (designated A1, A2, A3, A4, AD2, AD1, B, C, and D, or fnA-D) which are missing in the shortest splice variant (TN.S). Fn3 contains the RGD and EIDGIELT sites, and fnD contains VFDNFVLK.

eral domains within the same or different types of component proteins. Hence individual domains or regions of matrix proteins can have actions that either mimic or differ from those of the parent molecule *(1,2)*.

The study of isolated domains has allowed the identification of short amino acid sequences that provide specific growth cues to neurons, e.g., signals to extend longer neurites *(3)*, or signals for guided neurite outgrowth *(4)*. These peptide fragments have the potential to be potent therapeutic reagents for the encouragement of axonal regeneration following brain or spinal cord injury. However, it must be stressed that in the absence of the appropriate type and number of receptors, the peptides will not generate any biological activity. It is the purpose of this review to discuss the current knowledge relating to biologically active protein fragments for CNS neurons contained within the amino acid sequences of extracellular matrix molecules, with emphasis given to the glycoproteins tenascin-C, laminin-1, and fibronectin. In addition, these peptides will be discussed in the context of their interactions with their identified receptors, in particular the integrins. Implications of such matrix/receptor interactions will also be examined in the context of designing strategies to improve regeneration in the CNS.

Tenascin-C

Our work has been directed toward the regulation of neuronal growth by tenascin-C, an extracellular matrix glycoprotein that is normally restricted in the CNS to sites of tissue development and regeneration. Tenascin-C exists as a family of alternatively spliced variants that differ only in their number of fibronectin type-III (FN-III) repeats. Structurally, all of the splice variants are comprised of six identical arms. Three arms are joined together at their N-termini to form half of the tenascin-C molecule, with the two halves joined by a disulfide bond at the central nodule (Fig. 1). Each arm is in turn comprised of a linear series of domains with homology to epidermal growth factor (EGF), followed by a series of domains

Table 1
Active Peptides Derived from Extracellular Matrix Glycoproteins and Their Receptors

Peptide	Molecular origin	Domain or region	Putative receptor	Reference(s)
VFDNFVLK	**Tenascin-C**	fnD	α7β1 integrin	*11*
EIDGIELT		fn3	α9β1 integrin	*18*
?		fnB-D	F3/contactin	*22*
?		fn6-8	α8β1 integrin	*7*
RGD		fn3	α8β1, αvβ3 integrins	*23,25–29*
RGD	**Fibronectin**	fn10	α3β1, α5β1, α7β1, α8β1, αvβ1, αvβ3, and αvβ6	*33–39*
FN-C/H I		fn14	?	*49,51*
FN-C/H-II		fn14	Unidentified heparin sulfate proteoglycan	*49,50,52*
CS1 (LDV)		IIICS	α4β1 integrin	*55,56*
FN-C/H V		C-terminus	α4β1 integrin	*58,59*
EDGIHEL		EDA	α4β1,α9β1 integrins	*61*
RDIAEIIKDI	**Laminin-1**	γ1 chain	Unidentified G-protein coupled receptor	*4,66–69*
IKVAV		α1 chain	β-amyloid precursor protein (APP)	*3,70,72–74*
YIGSR		#β1 chain	67 kDa YIGSR-binding protein	*76*
IKLLI		α1 chain	α3β1 integrin	*79*
RGD		α1 chain	α6β1, αvβ3 integrins	*81,84*
RKRLQVQLSIRT		α1 chain	?	*85*
?		E8 fragment	α7β1 integrin	*20,21*
KNRLTIELEVRT	**Laminin-2**	α1 chain	?	*85*
LRE	**s-laminin**	α2 chain	?	*87,88*

with homology to FN-III repeats, followed by a terminal fibrinogen knob. All variants have eight universal FN-III repeats; the largest tenascin-C splice variant differs from the smallest by the inclusion of a series of additional alternatively spliced repeats between universal FN-III repeats 6 (fn6) and 8 (fn8).

Regulation of neuronal growth by intact tenascin-C has been ascribed to several different domains of the molecule *(1,2,5,6).* For example, large and small splice variants of tenascin-C both form boundaries to growth-cone advance, attributable to the EGF repeats *(1,6).* Adhesion of peripheral neurons to tenascin-C is mediated by fn3, whereas adhesion of both peripheral and central neurons is mediated by fn6 *(5).* Neurite-outgrowth promotion has been mapped to the universal FN-III repeats 6-8 (fn6-8) *(2,6,7)* as well as to the alternatively spliced Fn-III repeats A-D (fnA-D) *(1,2,8,9),* and more specifically to fn6 *(1,5)* and fnD *(1,2).* The activity of some (fn3, fnD) but not all of these domains has been mapped to specific peptide sequences, as will be discussed below (and is summarized in Table I).

Research in our laboratory has focused on the growth-inducing actions of fnA-D in vitro *(10,11).* FnA-D promotes neurite outgrowth from a variety of CNS neurons as part of tenascin-C, and also when expressed by itself as a recombinant protein *(2,11).* Significantly, periods of cell and neurite motility in the developing CNS are closely correlated with expression of the large but not small tenascin-C, suggesting that fnA-D regulates axonal growth in vivo as well *(12,13).* FnA-D also provides permissive neurite guidance cues, mapped to fnC, that are distinct from its neurite-outgrowth promoting cues in fnD *(10).* Thus neurites given a choice at a poly-L-lysine (PLL)/fnA-D

interface *(10)* or a PLL/fnC interface (S. Meiners, unpublished data) demonstrates a significant preference for fnA-D andfnC, whereas no preference is observed for fnD. Hence the alternatively spliced region of tenascin-C may play a role in axon guidance in addition to an axon growth-promoting function during embryogenesis. Based on these results, we have hypothesized that exogenous fnA-D might be useful to encourage directed axonal regrowth following injury *(10,11).* Furthermore, the use of shorter peptides derived from fnD and fnC, respectively, might provide an effective means to specifically increase extension of damaged neurites, or to guide the regrowth of axons across the terrain of the glial scar.

We have recently turned our attention to the identification of biologically active peptides derived from fnA-D and have demonstrated that a short amino acid sequence in fnD derived from human tenascin-C, VFDNFVLK, is necessary and sufficient for outgrowth promotion from cerebellar granule neurons *(11).* Moreover, the conserved amino acids FD and FV are critical for activity. Site-directed mutagenesis and antibody-blocking experiments showed that this peptide is active by itself and also in the context of fnD. VFDNFVLK overcomes the inhibition induced by chondroitin sulfate proteoglycans on neurite outgrowth, which is significant in that chondroitin sulfate proteoglycans are upregulated in glial scars *(14)* and are considered to be major impediments to axonal regeneration following brain *(15)* and spinal cord *(16)* trauma. These results imply that peptides containing the sequence VFDNFVLK may find applicability as reagents, independent from fnA-D, to increase axonal regeneration in vivo. Work is in progress to identify an active peptide in fnC that functions as a cue for neurite guidance.

In light of these observations, the question that next arises concerns the identity of the neuronal receptor that interacts with VFDNFVLK. A comparison of VFDNFVLK in fnD and the corresponding sequence EIDGIELT in fn3, for which a receptor has been identified *(18),* implicates a β1 integrin as a candidate receptor. The structure of fn3 has been reported *(17),* and EIDGIELT includes portions of an exposed loop and adjacent beta strand. D in the exposed loop and E in the adjacent beta strand are required for binding to the integrin α9β1 *(18).* By analogy, it is highly likely that FD found in VFDNFVLK is localized on an exposed loop in fnD for an interaction with neurons, whereas FV is likely to be positioned on a semi-buried beta strand, where it may lend conformational stability rather than binding directly to neurons. However, it is unlikely that VFDNFVLK binds to the α9β1 integrin, because α9β1 has not been detected in brain *(19),* and we have been unable to detect it in the cultured cerebellar granule neurons used in our work. Instead, our data suggests that VFDNFVLK interacts with the α7β1 integrin, a laminin-1 receptor involved in neuromuscular *(20)* and myotendinous junctions *(21),* to promote neurite outgrowth from cerebellar granule neurons (M.L.T. Mercado and S. Meiners, unpublished data). The peptide sequence that α7β1 binds to in laminin-1 is unknown, but the latter does not contain a sequence homologous to VFDNFVLK despite the fact that it also interacts with α7β1 to facilitate process extension from cerebellar granule neurons (M.L.T. Mercado and S. Meiners, unpublished data). This indicates that α7β1 may bind to more than one peptide sequence, or that amino acids on nonlinear parts of laminin-1 come together to form an active site homologous to VFDNFVLK.

VFDNFVLK is likely not the only sequence responsible for neurite outgrowth by the alternatively spliced region of tenascin-C. Therefore other peptides may exist with potential utility to increase axonal outgrowth following CNS trauma. Recombinant proteins corresponding to the tenascin-C FN-III repeat pairs BD and D6 were both shown to facilitate process extension from embryonic hippocampal neurons in vitro *(1,22).* Antibodies to the cell adhesion molecule F3/contactin of the Ig superfamily blocked the actions of fnB-D but not fnD-6, and F3/contactin bound to fnB-D but not to fnD-6. This suggests that fnB-D influences process extension via the F3/contactin receptor, and that an

as yet unmapped site in fnB is required for neurite outgrowth and receptor binding. This stands in marked contrast to our previous results, which indicated that VFDNFVLK in fnD represents the only active site for neurite outgrowth in fnA-D *(11),* and by extension, it would appear, in fnB-D. Because cerebellar granule neurons express F3/contactin *(22),* our results cannot be explained by the lack of an appropriate receptor for fnB. On the other hand, the recombinant fnA-D protein used in our study included the fnC domain, whereas the fnB-D protein used in the F3/contactin study lacked fnC. Accordingly, it is possible that amino acids in fnB and fnD form an active site for neurite extension and F3/contactin binding only when the two FN-III repeats are adjacent to one another, a spatial coordination that cannot occur in the presence of fnC. Of interest, splice variants containing fnB-D but not fnC represent a large proportion of tenascin-C isoforms present in the embryonic hippocampus, whereas the fnC repeat is inserted in between fnB and fnD postnatally *(22).* Thus, fnB-D interactions with F3/contactin may represent a unique mechanism to regulate axonal extension during embryonic as opposed to early postnatal development of the brain.

As mentioned above, fn6–8 represents another tenascin-C region that avidly promotes neurite outgrowth from cerebral cortical, cerebellar granule, and hippocampal neurons in vitro in the context of tenascin-C and also as a substrate-bound recombinant protein *(2,6).* The permissive effect of fn6-8 is replicated by fn6 *(1,5)* but not by fn7-8 *(1),* demonstrating that the outgrowth-promoting site likely resides in fn6. However, VFDNFVLK is found only in fnD and is not shared by fn6, fn7, or fn8. Experiments with function-blocking antibodies to integrin subunits indicated that fn6–8 facilitates neuronal process extension via the α8β1 integrin receptor *(7).* In addition to interacting with fn6-8 to promote neurite outgrowth, α8β1 also binds to the RGD motif in fibronectin, vitronectin, and the fn3 repeat of tenascin-C *(23),* as well as the RGD motif in osteopontin *(24),* to mediate adhesion of kidney cells. However, fn6-8 does not contain an RGD sequence, and tenascin-C fragments containing the RGD sequence do not facilitate neuronal process extension from CNS neurons *(1,2,6).* Hence the neurite-outgrowth promoting/α8β1-binding sequence in fn6 remains to be determined.

Some controversy remains about whether the RGD site in the tenascin-C fn3 domain is in fact functional in the intact molecule or is cryptic, i.e., revealed only in shorter fragments. The RGD site interacts with the integrin αvβ3 as well as the integrin α8β1 to promote cellular adhesion *(25,26),* and receptor/ligand investigations for both αvβ3 and α8β1 integrin/RGD interactions have provided conflicting data on this issue. For example, α8-transfected cells adhered to intact tenascin-C and tenascin-C fragments containing RGD *(23),* suggesting that the site is functional in the native molecule. Furthermore, RGD-containing peptides inhibited αvβ3 integrin-mediated adhesion of endothelial cells to native tenascin-C *(25).* On the other hand, the fn3 repeat of tenascin-C supported strong adhesion and spreading of endothelial cells as an isolated domain, whereas a longer tenascin-C fragment containing fn2 as well as fn3 supported less adhesion *(25).* This was suggested to be due to partial blockade of RGD by the adjoining FN-III repeat. Moreover, a recombinant soluble heterodimer consisting of the extracellular domains of α8 and β1 integrin chains bound avidly to the isolated fn3 repeat, but not to full length tensacin-C and longer fragments containing fn3 *(27).* It seems likely, then, that the RGD motif in tenascin-C is partially, but not wholly, obstructed in the intact molecule.

Nevertheless, recent investigations *(28)* have indirectly supported a function of the integrin αvβ3 in neurons, and potentially its tenascin-C RGD peptide ligand. Neuroblastoma cells were encouraged to differentiate into neurons by treatment with 12-0-tetradecanoylphorbol-13-acetate (TPA) or retinoic acid (RA). TPA and RA both induced secretion of large and small splice variants of tenascin-C by the cells, upregulation of αv integrin subunit expression, increased cellular adhesion, and

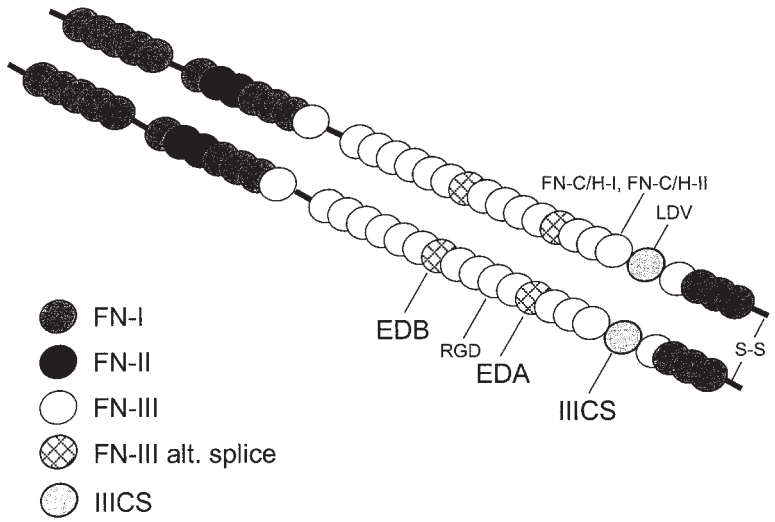

Fig. 2. Multi-domain structure of fibronectin. Each alternatively spliced variant of the fibronectin family contains 12 universal FN-I repeats, 2 universal FN-II repeats, and 15 universal FN-III repeats. The positions of the alternatively spliced EDB, EDA, and IIICS domains are indicated. Two fibronectin monomers can be joined by disulfide bonds at their C-termini to form a covalently linked dimer. Universal FN-III repeat 10 contains the RGD site, whereas universal FN-III repeat 14 contains the FN-C/H-I (YEKPSPPREVVPPRPGV) and FN-C/H-II (KNNQKSEPLIGKKT) amino acid sequences. The IIICS domain contains the LDV site.

enhanced neurite outgrowth. Cells exposed to TPA also demonstrated enhanced expression of α2 and β1 integrin subunits, suggestive of a role for integrin α2β1, which binds to an as yet undefined site in tenascin-C *(26).* Hence differentiating neural cells may produce their own substrate molecules and concomitant receptors for enhanced adhesion and neurite outgrowth. Another study *(29)* provides evidence that the αv integrin functions in the CNS under pathological conditions to facilitate survival of tumor cells by promoting adhesion to tenascin-C and vitronectin. In this study, the EMD 121974 αv integrin antagonist, a cyclic RGD-containing penta-peptide, inhibited brain-tumor growth by detaching the tumor cells from tenascin-C and vitronectin and subsequently inducing apoptosis. This represents an example of a clinical use of a tenascin-C-directed peptide in cancer therapy and provides optimism that similar peptides may be exploited in therapeutic strategies to improve axonal regeneration.

Fibronectin

Fibronectin is present in the developing mammalian CNS at areas of axon extension *(30)* and promotes neuronal adhesion and neurite outgrowth in vitro *(31).* Like tenascin-C, fibronectin exists as a family of alternatively spliced variants. All variants contain twelve universal FN-I repeats, which make up the N-terminal and C-terminal regions of the molecule; two universal FN-II repeats; and fifteen universal FN-III repeats (Fig. 2). Fibronectin variants are generated by alternative splicing of FN-III repeats EDB (also called EIIIB or EDII) and EDA (also called EIIIA or EDI), as well as alternative splicing of type III connecting segment IIICS (also called V, for variable). The resulting fibronectin monomers can form two disulfide bonds at their C-termini, producing a covalently-linked dimer.

Fibronectin has already demonstrated promise as a conduit material for nerve regeneration following peripheral nervous system

injury *(32)*. Oriented strands of fibronectin were used in comparison with autologous nerve grafts to bridge a 1 cm lesion in rat sciatic nerve. While the nerve grafts originally supported the highest extent of axonal regeneration, the fibronectin and nerve grafts contained similar numbers of regenerating peripheral neuronal processes after 15 d. Hence oriented patterns of fibronectin, and potentially neurite-outgrowth promoting peptides derived from fibronectin, may encourage axonal regeneration in the CNS as well.

The tri-peptide RGD, contained within the tenth FN-III repeat, represents perhaps the most studied short amino acid sequence in fibronectin. RGD binds to the integrins α3β1 *(33)*, α5β1 *(34)*, α7β1 *(35)*, α8β1 *(36)*, αvβ1 *(37)*, αvβ3 *(38)*, and αvβ6 *(39)*. Furthermore, RGD and a second site within the ninth FN-III repeat interact with the integrin αIIbβ3 *(40)*, and RGD synergizes with sites within the eighth and ninth FN-III repeats to interact with the integrin α5β1 *(41,42)*. Since the RGD site binds to a large number of diverse integrins, and integrin subtypes can activate distinct signal transduction pathways downstream of the same extracellular ligand via their cytoplasmic domains *(43)*, it is probable that RGD can differentially effect neuronal growth depending on cell type-specific expression of a particular RGD-binding integrin(s).

As a case in point, RGD appears to differentially influence adhesion and outgrowth of central and peripheral nervous system neurons. For example, both parameters were reduced when neurons were plated on intact fibronectin in the presence of soluble RGDS peptide *(44)*, but the inhibition was much greater for peripheral than for central neurons. Similarly, no promotion of neurite outgrowth was observed from central neurons adhered to RGD-containing tenascin-C fragments *(1,2,6)*, whereas peripherally derived dorsal root ganglion neurons elaborated long processes when plated on the tenascin-C's fn3 repeat in an RGD-dependent manner *(5)*. These results suggest that both central and peripheral neurons have receptors for RGD, but that the relative importance of the site is greater for peripheral neurons. It is possible that peripheral as opposed to central neurons respond to the RGD site in fibronectin and tenascin-C using an integrin that either demonstrates a higher affinity for the sequence, or that contains a cytoplasmic tail capable of enhanced RGD-dependent signal transduction for promoting adhesion and outgrowth responses. The α8β1 integrin is one such candidate, since it binds to the RGD sequence found in both fibronectin and tenascin-C *(23)* and mediates the fibronectin-dependent neurite outgrowth responses of dorsal root ganglion neurons *(36)*. On the other hand, central neurons also express α8β1 *(45)*, yet they do not grow longer processes in response to RGD. Regardless of the mechanism responsible for the differential regulation of peripheral vs central neuronal growth by RGD, the practical implication is that RGD-containing fibronectin (and tenascin-C) fragments may have a greater potential to promote the regrowth of injured peripheral as opposed to CNS neurons.

Additional studies suggest that the RGD site in fibronectin, and hence RGD-containing fibronectin peptides, have pronounced activity for peripherally derived neurons. In one such study, divalent manganese induced neurite outgrowth in rat PC12 pheochromocytoma cells when the cells were grown in serum-free defined medium on surfaces coated with fibronectin, but not on untreated surfaces or those coated with poly-D-lysine *(46)*. GRGDS almost completely inhibited manganese-induced neurite outgrowth on fibronectin *(46,47)*, indicating involvement of an RGD-dependent fibronectin/integrin interaction. Furthermore, adherence of neuroblastoma cells to an RGD-containing fragment of fibronectin and the subsequent neurite extension were completely inhibited by RGDS, suggesting that an RGD-binding integrin also mediates these processes *(48)*. In contrast, RGDS only partially inhibited outgrowth from neuroblastoma cells on intact fibronectin, revealing at least one additional active site for peripheral neurite dif-

ferentiation, and perhaps central neurite differentiation as well, outside of the RGD-containing fragment.

Later investigations indicated that the additional active sites(s) most likely resided in the 33 kDa C-terminal cell and heparin-binding fragment of fibronectin. This fragment is a strong promoter of RGD-independent cellular adhesion *(49)* and neurite outgrowth from CNS neurons *(50).* Two heparin-binding peptides that promoted adhesion of melanoma cells were originally identified in this fragment, FN-C/H I (YEKPGSPPREVVPRPRPGV) and FN-C/H-II (KNNQKSEPLIGRKKT) *(49).* FN-C/H I and antibodies directed against FN-C/H I significantly inhibited melanoma-cell adhesion and heparin binding, respectively, to native fibronectin. FN-C/H II, on the other hand, was shown to mediate neuronal cell adhesion and neurite outgrowth, in addition to heparin binding and melanoma cell-adhesion, via interactions with a neuronal heparin sulfate proteoglycan *(50).* The active site in FN-C/H II was subsequently mapped to LIGRKK *(52).* Significantly, an all D-configuration analogue of FN-C/H II was shown to have similar activity to the native all-L sequence *(53),* which is important for potential clinical applications of this peptide in strategies to improve axonal regeneration in the CNS.

As with RGD, FN-C/H II appears to have a differential impact on the growth of central vs peripheral nervous system neurons. Central and peripheral neurons were cultured on substrates coated with FN-C/H I, FN-C/H-II, or CS1 *(54).* CS1 *(55)* is a major cell-attachment site derived from the alternatively spliced type III connecting segment (IIICS) of fibronectin and contains the α4β1 integrin-binding sequence LDV *(56).* Neurite outgrowth from spinal cord neurons, derived from the CNS, was the most extensive on FN-C/H-II. In contrast, neurite outgrowth from dorsal root ganglion neurons, derived from the peripheral nervous system, was optimal on CS1 *(54).* Furthermore, neurite outgrowth from spinal cord neurons was inhibited to a much greater extent by soluble heparin than by antibodies against β1 integrin chain, indicating that process extension from spinal cord-derived neurons may be mediated by an FN-C/H-II-binding neuronal heparin sulfate proteoglycan. Hence FN-C/H-II may find more applicability than CS1 as a reagent to increase axonal regrowth of central neurons in the injured spinal cord.

On the other hand, CS1 has demonstrated potential efficacy as a therapeutic agent for neuronal protection from cerebral ischemia, although the site of direct actions of the peptide were probably on leukocytes as opposed to on neurons *(57).* Rats were subjected to transient ischemia without treatment, or treatment with RGD peptide, CS1 peptide, FN-C/H V peptide, or scrambled FN-C/H V peptide. [FN-C/H V (WQPPRARI), like FN-C/H I and II, is derived from the C-terminal heparin-binding domain of fibronectin *(58)* and has, along with CS1, been implicated as a ligand for the α4β1 integrin *(59).*] Rats treated with either CS1 or FN-C/H V showed a significant decrease in the amount of leukocyte infiltration and infarct size, as well as a significant improvement in neurological grade, when compared to untreated rats or those treated with RGD or scrambled FN-C/H V. Because leukocytes play an important role in the development of ischemic brain damage, these results suggest that leukocytic α4β1 integrin interactions with FN-C/H V or CS1 may lead to leukocyte arrest and subsequent neuronal protection.

The alternatively spliced EDA FN-III repeat represents another α4β1 integin-binding region within fibronectin. EDA also serves as a ligand for the α9β1 integrin, which is closely related to α4β1 based on amino acid sequence *(60).* EDA/α4β1 and EDA/α9β1 integrin interactions both mediate cell adhesion to fibronectin *(61),* establishing a new role for alternative splicing of fibronectin. The recognition site within EDA for α4β1 and α9β1 was mapped to EDGIHEL in an extended loop region of the FN-III repeat *(61)*, which is similar to the α9β1 integrin-binding site EIDGIELT in the third universal FN-III repeat of tenascin-C *(18).* As mentioned above, α9 integrin has not been detected in CNS neurons *(19),* although mRNA transcripts for the

α4 integrin have been found in several regions of the brain *(45).* Therefore, EDGIHEL might play a role in regenerative strategies following brain trauma by influencing adhesion or other growth regulatory functions of CNS neurons via interactions with α4β1 integrin.

Laminin-1

Laminins form a major component of the basement membrane, and they are also found in the peripheral and central nervous system during development and after injury *(62).* The laminins consist of a family of heterotrimeric proteins (each with an α, β, and γ chain, each coded by a different gene). While several different laminins have been identified based on the assembly of different α (α1–5), β (β1 to 3) and γ (γ1–3) chains, laminin-1 (α1-β1-γ1) is the predominant species in the CNS, and it is spatially and temporally associated with neuronal migration and axonal growth in vivo *(63).* Structurally, the laminin-1 molecule resembles a cruciform. The C-termini of its three chains come together to form the rod-like coiled-coil structure, which represents the long arm of laminins; the globular domains are formed by the most distal C-terminal region of the α1 chain (Fig. 3). The short arms are formed by the N-termini of the α, β, and γ chains and feature a series of LN, LE, and L4 domains.

As with tenascin-C and fibronectin, specific neuronal growth regulatory functions can be ascribed to laminin-1. For example, laminin-1, like the fnA-D region of tenascin-C, promotes directional outgrowth of neurites on patterned substrates *(64)* and also promotes neurite outgrowth when presented as a uniform substrate *(65).* This, along with its in vivo localization, suggests that laminin-1 is a molecular cue for axonal growth and guidance in the developing CNS. This also suggests that laminin-1-derived peptides may be useful reagents to facilitate the same functions in the injured brain and spinal cord for the repair of damaged neurons. The search for biologically active peptides in laminin-1 has been extensive, and a comparatively larger number of sequences demonstrating the capacity to regulate neuronal growth has been identified for laminin-1 as opposed to tenascin-C or fibronectin.

The RDIAEIIKDI sequence derived from the C-terminal end of the γ1 chain forms an important active site for neuronal growth in laminin-1 *(66).* When cerebellar granule neurons were cultured on laminin-1, a synthetic RDIAEIIKDI peptide competed with the native molecule to inhibit neuronal attachment and neurite outgrowth *(66).* The peptide also supplied both outgrowth and guidance cues for hippocampal neurites in vitro *(4).* The neuronal receptor for RDIAEIIKDI is unknown, but the peptide seems to exert its effects on CNS neurons via an as yet unidentified G-protein coupled receptor *(67).* RDIAEIIKDI also apparently facilitates neuronal migration, since antibodies against RDIAEIIKDI-containing peptides completely inhibited migration of mouse cerebellar granule neurons in a cell culture model *(68).* Subsequent investigations demonstrated that the tri-peptide KDI facilitates process extension and induced electrical currents in cerebellar granule neurons *(67)* and neocortical neurons *(69),* suggesting that KDI forms the active site in the deca-peptide. However, RDIAEIIKDI was shown to be toxic to neurons at high concentrations *(67),* indicating that it may not be suitable as a therapeutic reagent for CNS injury.

Other investigations have revealed that IKVAV, located at the C-terminal end of the long arm of laminin-1, might be a more promising candidate for regenerative strategies. IKVAV promotes neurite outgrowth from central and peripheral neurons in the context of laminin-1 and also by itself, as a synthetic peptide *(3,70).* An all D-configuration peptide containing the IKVAV sequence was shown to have similar biological activity to the native all-L peptide, suggesting that the modified compound is potentially useful for clinical applications. Furthermore, no neuronal toxicity was associated with IKVAV *(71).* Although the neuronal receptor for IKVAV was not identified in these studies, the peptide was shown in later studies to interact with a 110 kDa

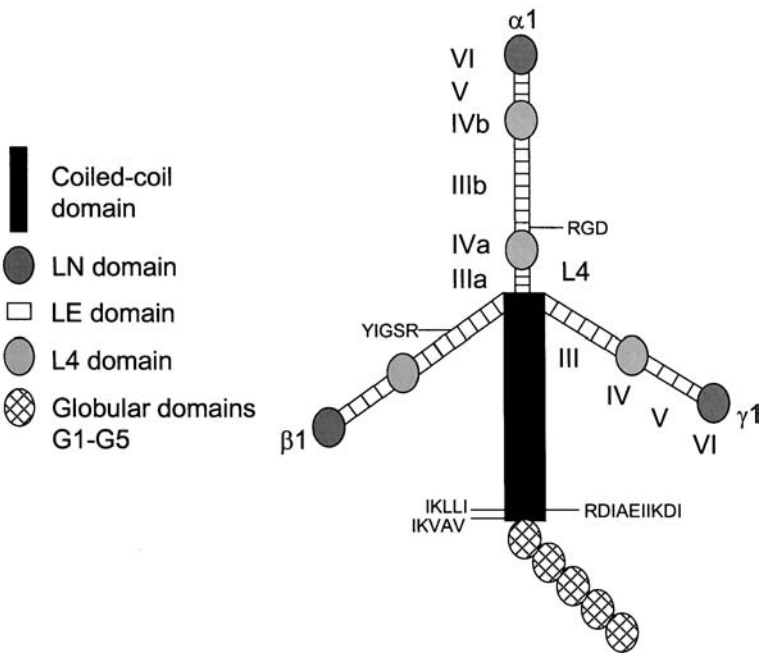

Fig. 3. Multi-domain structure of laminin-1. Laminin-1 is comprised of 3 protein chains (α1, β1, and γ1) that interact to form a coiled-coil domain at their C-termini. The globular domains (G) are formed by the most distal C-terminal portion of the α1 chain. The positions of the LN, LE, L4, and G domains are indicated. The RDIAEI-IKDI site is within the γ1 chain at the C-terminal end of the coiled-coil domain, and the IKVAV and IKLLI sites are located within the α1 chain at the C-terminal end of the coiled-coil domain. YIGSR is found in the β1 chain, and RGD lies within the IIIb region of the α1 chain.

laminin-1-binding plasmalemmal protein, LBP110 *(72,73)*, which reportedly corresponds to β-amyloid precursor protein (APP) *(74)*. On the other hand, IKVAV is not recognized by integrins of the β1 family *(72)*. In other studies, IKVAV-containing peptides but not antibodies directed against laminin-1 perturbed neurite outgrowth of dorsal root ganglion neurons on cryostat sections of neonatal rat spinal cord *(75)*. As suggested, this indicates that the developing CNS may contain neurite outgrowth promoting molecules distinct from laminin-1 that share IKVAV or a related sequence, or alternatively that IKVAV adopts a more active conformation independent of the laminin-1 molecule.

IKVAV as well as YIGSR, a laminin-1-derived peptide found on the β1 chain *(76)*, have received attention in investigations on the utility of peptide-modified surfaces for cellular growth. Peptides containing the sequences YIGSR and IKVAV were covalently bound to hydroxylated fluorinated ethylene propylene films. Films containing YIGSR supported enhanced attachment of neuronal cells *(77)*, perhaps through interactions with a neuronal receptor homologous to the 67 kDa YIGSR-binding protein identified in epithelial cells *(76)*. Films containing IKVAV supported enhanced attachment as well as neurite outgrowth *(77)*. Subsequent studies demonstrated that surfaces modified with *both* IKVAV and YIGSR yielded a better cellular response from hippocampal neurons (as determined by increased neurite length and number of adherent cells) than did films modified with either

peptide alone *(78).* Hence, IKVAV and YIGSR may allow for the design of a biomaterial that can control neuronal attachment and differentiation following CNS injury, and mimic the attractive cues to which axons respond.

IKLLI, like IKVAV, represents another potentially valuable neurite-outgrowth promoting reagent derived from the coiled-coil region of the α1 chain of laminin-1. Unlike IKVAV, IKLLI interacts with the α3β1 integrin *(79).* IKLLI is apparently not the only site in laminin-1 that binds to α3β1, since KQNCLSSRASFRGCVRNLRLSR derived from the globular domain of the α1 chain was shown to bind α3β1 in affinity chromatography experiments and eluted bound α3β1 from laminin-1 *(80).* However, in contrast to IKLLI, no activity was observed for KQNCLSSRASFRGCVRNLRLSR on neuronal growth. Antibodies against IKLLI-containing peptides partially impaired adhesion and process extension from PC12 cells on laminin-1 and IKLLI, demonstrating that this sequence is also functional in the intact protein *(79).* Investigations demonstrating the activity of IKLLI *(79)* and IKVAV *(3)* in native laminin-1 indicate that the coiled-coil region is relatively flexible, providing a means for these sites to be exposed to cells.

Laminin-1 also contains the classical RGD integrin-binding motif in the IIIb region of the α1 chain, and studies with neuroblastoma cells indicate that this site might be active for CNS neurons. Synthetic peptides containing the RGD sequence and its flanking amino acids were active in promoting cell adhesion and neurite outgrowth from centrally derived neuroblastoma cells via the α6β1 integrin, but only when coupled to keyhole limpet haemocyanin (KLH) *(81).* The fact that the peptides did not facilitate process extension by themselves suggests that they may not independently adopt an appropriate conformation for receptor binding, but rather must be presented to the receptor in the context of a larger protein. This is not surprising given that the amino acid sequence, structure (cyclic/linear), and conformation of RGD peptides appear to be critical features influencing ligand interaction with integrin *(82).*

On the other hand, the RGD-containing peptides blocked neuroblastoma cell adhesion to laminin-1 *(81),* suggesting that RGD represents an active site in the native molecule. These results are in contrast to those from an earlier study, which reported that the RGD site is cryptic in laminin-1 and only available after proteolysis *(83).* Similarly, other investigations showed that a laminin-1 fragment containing the globular domain of the α1 chain bound to αvβ3 integrin only in the presence of the adjacent RGD site, although with lower affinity than did a laminin-1 fragment with a fully exposed RGD site *(84).* Taken together, the data suggests that the RGD site in laminin-1, as in tenascin-C, is partially but not fully masked. Regardless, strategically designed substrates which include laminin-1-derived peptides containing the RGD site in an appropriate conformation may find a role in tissue engineering approaches to improve axonal regrowth.

Certain peptides in laminin-1 have been shown to be active for promotion of neurite outgrowth from some neuronal cell types but not others. For example, the sequences RKRLQVQLSIRT from the C-terminal globular domain of the α1 chain of laminin-1 and KNRLTIELEVRT from the corresponding region of laminin-2 supported neurite outgrowth in two out of three neuronal cell lines tested *(85).* Later investigations identified a series of 23 peptides derived from the α1, β1, and γ1 chains that were active in encouraging process extension from two neuronal cell lines as well as cerebellar granule neurons, and a series of 25 additional peptides that were active for some but not all of the cell types tested *(86).* This may be due to the presence of different laminin-1 receptors on different classes of neurons. Certain of these peptides (e.g., GGFLKYTVSYDI, LSNIDYILIKAS, TNAVGYSVYDIS) competed with intact laminin-1, suggesting that they represent active sites in the molecule. Hence laminin-1 has multiple active sites for neurite outgrowth, many of which are specific for particular cell types. This in turn suggests that particular laminin-1-derived peptides might find applica-

bility as reagents to increase neurite regeneration in different parts of the nervous system.

A special mention must be made of the LRE stop signal in the β2 chain of s-laminin, a homologue of laminin concentrated in the synaptic cleft of the neuromuscular junction *(87,88).* LRE promoted adhesion of motoneurons in the spinal cord to s-laminin and to s-laminin-rich basal laminae in tissue sections in an integrin-independent manner *(87).* Furthermore, in experiments using mixed substrates, LRE also inhibited neurite outgrowth promoted by laminin-1 both in the context of a SRE-containing s-laminin fragment and as a free tripeptide *(88).* Because the signal was selective for motor neurons in the spinal cord, LRE was suggested to be a contact-dependent signal that plays a role in motor neuron synapse formation.

Other work, however, showed that native s-laminin promotes rather than inhibits neurite outgrowth *(89),* and that LRE may differentially impact neuronal growth depending on structural context. LRE is found in the coiled-coil region formed by the α, β, and γ chains in the intact protein. A recombinant randomly coiled LRE peptide inhibited neurite outgrowth from motor neurons in this study, whereas a small recombinant triple coiled-coil protein did not. Hence LRE may not inhibit process extension in vivo. Regardless of its physiological function, it may find a role as a reagent for patterned substrates in tissue engineering strategies to increase axonal regrowth: alternating lanes of LRE and a growth promoting peptide such as VFDNFVLK from tenascin-C or IKVAV from laminin-1 in a graft could serve to confine neurites to specific paths; i.e., across a glial scar that forms after CNS injury.

Implications for Central Nervous System (CNS) Regeneration

Despite notable exceptions outlined above, it is clear that the neuronal growth regulatory properties of tenascin-C, fibronectin, and laminin-1-derived peptides are mediated in large part through neuronal integrin receptors. This in turn has important implications for the utility of these peptides as reagents in strategies to encourage regeneration in the adult CNS. For example, neurons in the developing CNS are capable of substantial neurite regeneration *(90),* whereas neurons in the adult CNS normally do not regenerate *(91).* We and others *(10,92,93)* have suggested that an inhibitory environment with a paucity of growth-promoting molecules, matrix-derived or otherwise, contributes to failed regeneration of adult central neurons. However, a reduced number of receptors for growth-promoting molecules on adult as compared to young neurons may also play a major role. In support of this idea, adult dorsal root ganglion neurons, which do not regenerate their central processes, demonstrated low expression of integrins and poor process extension on laminin-1 and fibronectin in comparison to early postnatal dorsal root ganglion neurons. Increasing the expression of α1 or α5 integrin subunit in adult neurons by adenovirus-mediated gene transfer resulted in greatly enhanced neurite outgrowth on laminin-1 and fibronectin, respectively *(94).* Hence maximal neurite regrowth in the injured adult CNS will probably involve a codependent strategy to increase expression of integrins and other key receptors in damaged neurons as suggested *(94)*, as well as the introduction of a regeneration cocktail of growth-promoting molecules such as extracellular matrix-derived peptides.

Like embryonic and early postnatal central neurons, adult peripheral neurons are capable of extensive neurite regeneration *(91).* Recently, elevated expression of α7β1 has been connected to the successful regrowth of damaged peripheral neuronal processes in mature mice *(95).* Peripheral nerve axotomy resulted in a large increase in immunoreactivity for both α7 and β1 integrin subunits on regenerating sensory and motor neurites at sites of axon–axon and axon–Schwann cell contacts. Moreover, α7 integrin subunit knockout mice demonstrated impaired axonal regrowth and delayed target reinnervation in the facial

nerve, suggesting that α7β1 is absolutely critical for regeneration. Given that laminin-1 and tenascin-C are both upregulated following peripheral nervous system injury at the same sites as α7β1 *(96–98),* and given that α7β1 apparently interacts with both VFDNFVLK in tenascin-C and an unknown site in laminin-1 (M. L. T. Mercado and S. Meiners, unpublished data), either matrix molecule could potentially serve as a ligand to promote neurite regrowth of peripheral neurons. In contrast, α7β1 integrin was not increased following central nerve injury, leaving open the question of whether low α7β1 expression is a limiting factor in CNS regeneration. It seems likely that manipulating the expression of this receptor in damaged adult central neurons might improve their ability to regenerate in response to reagents such as VFDNFVLK or laminin-1 derived peptides.

Other integrins in addition to α7β1 have been shown to be upregulated under conditions in which neuronal regeneration occurs. Expression of α4 integrin is increased in neuronal cell bodies and growth cones in the sciatic nerve during peripheral nervous system regeneration *(99).* Fibronectin splice variants containing the alternatively spliced domains EDA, EDB, and IIICS are also upregulated following peripheral injury, where they have been suggested to contribute to the favorable regenerative environment of the peripheral nervous system *(100).* Since EDA and IIICS interact with α4β1 integrin via the sequences EDGIHEL and LDV, respectively *(56,61),* the number of fibronectins that potentially bind to α4 integins is upregulated. Significantly, the interaction of fibronectin fragments containing the α4 binding site with α4 integrin increases neurite outgrowth from dorsal root ganglion neurons in vitro. Furthermore, PC12 cells grow longer processes on fibronectin following α4 expression *(99).* α4 is therefore apparently an important player for regeneration of peripheral neurons, which indicates that increasing the expression of α4 in CNS neurons might increase their regenerative response to fibronectin and fibronectin-derived EDGIHEL and LDV peptides in a regeneration cocktail.

The integrins α3β1, which binds IKLLI in laminin-1 *(79)* and RGD in fibronectin *(33),* and α6β1, which binds RGD in laminin-1 *(81),* are similarly upregulated in peripheral neurons in a successful model of axonal regeneration *(101).* Chondroitin sulfate proteoglycans inhibit neurite outgrowth and form barriers to growth cone advance in the developing as well as the regenerating nervous system *(15,102,103).* However, a recent study indicates that embryonic peripheral neurons can adapt to proteoglycan inhibition by increasing their expression of receptors for growth promoting matrix molecules such as laminin-1 *(101).* In this study, embryonic dorsal root ganglion neurons cultured on laminin-1 in combination with the proteoglycan aggrecan increased RNA and surface protein for the laminin-1 integrin receptors α3β1 and α6β1. Increased integrin expression paralleled increases in neuronal adhesion and neurite outgrowth. Directly increasing integrin expression by adenoviral infection to levels similar to those seen in the adapted neurons also eliminated aggrecan inhibition of neuronal growth. The ability of embryonic neurons to adapt to aggrecan by upregulating integrin expression *(101)* parallels the ability of adult peripheral neurons to regenerate their processes *(95,99)* in the presence of inhibitory proteoglycans through upregulation of integrin expression. Moreover, as previously suggested *(101),* these results again indicate that manipulation of integrin expression in central neurons might provide a mechanism for increasing their regenerative potential following brain or spinal cord trauma.

Conclusions

In conclusion, we have outlined a number of amino acid sequences contained in the extracellular matrix proteins tenascin-C, fibronectin, and laminin-1 with demonstrated growth regulatory functions for CNS neurons, as well as the neuronal receptors with which they interact. We have suggested that these peptides

might serve as reagents to enhance axonal regrowth following CNS injury. Since integrins represent the major class of receptors for neuronal growth promoting matrix peptides, their expression in neurons has profound consequences for their successful use in therapeutic strategies. This is most significant in light of the observations showing that integrins are upregulated in peripheral but not central neurons under conditions in which regeneration and adaptation to chondroitin sulfate proteoglycans occur *(95,99,101).* Therefore, manipulation of the expression of integrins in damaged adult CNS neurons may be a vital companion approach to improve their ability to regenerate in response to reagents such as VFDNFVLK, IKLLI, RGD, and others.

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